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Biphasic glucocorticoid rhythm in one month old infants: reflection of a developing HPA-axis?

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Abstract

Context: The hypothalamus-pituitary-adrenal (HPA) axis displays a diurnal rhythm. However, little is known about its development in early life.

Objective: To describe HPA-axis activity and study possible influencing factors in 1-month-old infants.

Design: Observational

Setting: Amsterdam UMC, location VUMC, and OLVG, Amsterdam

Participants: Fifty-five mother-infant pairs

Interventions: Collection of breastmilk and infants' saliva 1 month postpartum for analysis of glucocorticoids (GCs; i.e., cortisol and cortisone) using LC-MS/MS

Main outcome measure: GC rhythm in infants' saliva, and associations with vulnerability for maternal psychological distress (increased Hospital Anxiety and Depression Scale (HADS) score or consultation at the Psychiatric-Obstetric-Pediatric (POP) clinic), season at sampling, sex and breastmilk GC rhythmicity, analyzed with Sigmaplot and regression analyses.

Results: A significant biphasic GC rhythm was detected in infants, with peaks at 6:53±1:01 (mean±SEM) and 18:36±1:49 for cortisol, and at 8:50±1:11 and 19:57±1:13 for cortisone. HADS-score, POP-consultation, season at sampling and sex were not associated with the infants' GC rhythm. Breastmilk cortisol maximum was positively associated with infants' cortisol area-under-the-curve (AUC) increase and maximum. Higher breastmilk cortisone AUCincrease, AUCground and maximum were associated with an earlier maximum in infants. Breastmilk and infant GC concentrations were associated between 6:00-9:00.

Conclusions: A biphasic GC rhythm, peaking in the morning and evening, was seen in 1-month-old infants at a group level. Breastmilk GC parameters might be associated with the infants' GC rhythm, possibly caused by a signaling effect of breastmilk GCs, or as an associative effect of increased mother-infant synchrony. These results contribute to an increased understanding of early-life HPA-axis development.

Précis

A biphasic GC rhythm was found in 1-month-old infants, possibly reflecting HPA-axis development.

Breastmilk GC rhythmicity could tentatively be associated with the infants' GC rhythm.

Introduction

In adults, the hypothalamus pituitary adrenal (HPA) axis displays a diurnal rhythm, peaking in the morning and with a nadir at night. However, it is not exactly clear when this adult-type rhythm is established in children, with studies reporting ages ranging from 2 weeks to 9 months in healthy infants.(1-9)

A rhythm in HPA-axis activity might already be present in the human fetus. Term neonates born in the afternoon through elective caesarian section appeared to have increased cortisol concentrations compared to neonates born during other times throughout the day.(10) Additionally, maternal estriol levels, partly reflecting dehydroandrostenedione (DHEAS) production by the fetal zone of the adrenal cortex, display a 24-hour rhythm during pregnancy inversely related to maternal cortisol levels.(11) Furthermore, several studies have shown data suggesting that a diurnal glucocorticoid (GC) rhythm is present from birth onward. Iwata et al. (2013) (12) described a diurnal cortisol rhythm peaking in the afternoon in newborns 2-11 days postpartum, while Spangler (1991) (8) found a biphasic pattern in neonates 2-7 days postpartum.

It is conceivable that an HPA-axis rhythm emerges prenatally, and continues to develop into an adult-type rhythm after birth, with a shift from a peak in the afternoon towards a morning peak. Currently, it is not clear which factors drive the development of an adult-type diurnal rhythm of the HPA-axis. Diurnal rhythms in general are mostly regulated by the suprachiasmatic nuclei (SCN), located in the anterior hypothalamus,(13,14) and its entrainment is predominantly dependent on exogenous time cues.(15) Indeed, light-dark cycles are an important regulator of SCN rhythmicity,(16) and have also been shown to influence infants' activity levels.(17) Moreover, maternal depressive disorders prior to or during pregnancy were associated with sleeping problems in infants,(18) while in adults psychopathology has been linked to changes in HPA-axis activity.(19,20) A twin study has previously concluded that environmental factors outweigh the genetic contribution to the development of an

HPA-axis rhythm.(9) However, which exogenous factors influence the development of an HPA-axis rhythm has not been studied yet.

Maternal activity has been associated with infant activity independent of exposure to light,(21) while formula milk with day/night nutrient levels in synchrony with the environment appeared to affect sleep patterns in infants.(22) Breastfeeding mothers have been shown to exhibit more touching and gazing behavior towards their infants, suggestive of more interactive behavior,(23) and these associations appear to be partly influenced by infant sex.(24) Breastmilk as well as breastfeeding itself might therefore also act as a possible contributor to the development of an HPA-axis rhythm. Additionally, breastmilk itself contains components which might aid in the development of an adult-type GC rhythm. For example, melatonin exhibits a strong diurnal pattern in breastmilk.(25) Similarly, our research group has previously shown that a diurnal rhythm of cortisol and cortisone is present in breastmilk, mirroring maternal HPA-axis activity.(26) In rats GCs were able to cross the intestinal epithelial barrier,(27) and earlier research in humans has shown that serum cortisol levels were 40% higher in infants who were breastfed (28).Moreover, cortisol levels in maternal and infant saliva were significantly correlated in breastfed infants, but not in formula-fed infants.(29) Accordingly, glucocorticoids in breastmilk might influence the process of HPA-axis rhythm development.

We therefore performed an exploratory study, aimed at assessing how some exogenous factors are associated with GC rhythmicity in infants, with a focus on the association with GC rhythmicity in breastmilk. GC levels were sampled at one month postpartum, since HPA-axis development into an adult-type rhythm appears to still be in progress at that time-point, while intra-uterine influences are likely to have disappeared. Both cortisol and cortisone were determined, since cortisone levels are higher compared to cortisol levels in both saliva and breastmilk, probably due to local conversion by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2),(30) and are therefore less likely to have a concentration below the lower limit of detection. Moreover, cortisone seems to be a more reliable biomarker compared to cortisol, at least in saliva and hair.(31,32) GCs in breastmilk, season at time

104 of sampling, maternal psychopathology and infant sex were explored as possible influencing factors,

105 with use of specialized rhythm analysis software.

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Methods

Study population

Between March 2016 and July 2017, mother-infant pairs were included from the general hospital OLVG as well as the academic Amsterdam UMC, location VUMC, both located in Amsterdam, as part of the Cortisol in Mother's Milk (CosMos) study. The primary aim of the study was to research the associations between breastmilk GC rhythmicity and the infant's own HPA-axis activity, behavior, and body composition. Women included at the OLVG were recruited at the Psychiatric Obstetric Pediatric (POP) outpatient clinic where they were monitored because of an increased risk for psychopathologic complaints. Inclusion criteria were: 1) born at term age (37-42 weeks), 2) normal birth weight (-2 to +2 SD), and 3) the intention to exclusively breastfeed for ≥ 3 months. Mother-infant pairs were excluded due to 1) major congenital anomalies, 2) multiple pregnancy, 3) pre-eclampsia or HELLP, 4) maternal alcohol consumption of >7 IU/week and/or 5) a fever (temperature >38.5°C) at time of GC sampling. Additionally, mothers were also excluded if they used medication other than "over the counter" drugs, except for anti-depressant use in the mother-infants pairs included at the OLVG.

Approval of the Medical Ethics Committee of the VUMC was obtained (protocol number 2015.524), and written informed consent was obtained from all participating mothers.

Data collection

Peripartum

Shortly after inclusion, within the first week postpartum, mothers filled in a questionnaire pertaining to their pregnancy and birth, as well as anthropometric and demographic data.

One month postpartum

One month postpartum (±5 days), mothers collected a portion of breastmilk (1-2 ml) before every feeding moment during a 24-hour period, with the use of a breast pump or via manual expression. In

order to minimize intra-individual differences, we requested mothers to use the same method for all their samples. Simultaneously, before feeding, they also collected their infant's saliva, using a SalivaBio Infant's Swab (exclusively from Salimetrics, State College, PA).

Milk and saliva was stored in the mother's freezer, and subsequently in the laboratory at -20°C for less than 3 months prior to analysis.

At time of sampling, mothers were also asked to fill in the Hospital Anxiety and Depression Scale (HADS) questionnaire, which assessed self-reported levels of depression and anxiety symptoms.(33,34) It contains 14 questions, with seven questions concerning depressive symptoms (HDS) and seven anxiety symptoms (HAS). Items are scored 0-3, and a score of ≥8 on one of the two subscales (HDS/HAS) is indicative of clinically relevant depression and/or anxiety symptoms.

Laboratory

Total cortisol and cortisone concentrations in breast milk were determined by isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS) as previously published.(35) In short, hexane washing was done thrice to remove lipids, after adding internal standards (13C3-labeled cortisol and 13C3-labeled cortisone). Then, Samples were extracted using Isolute plates (Biotage, Uppsala, Sweden) and analyzed by LC-MS/MS (Acquity with Quattro Premier XE, Milford MA, USA, Waters Corporation). The intra-assay coefficients of variation (CV%) were 4 and 5% for cortisol levels of 7 and 23 nmol/L, and 5% for cortisone levels of 8 and 33 nmol/L for LC-MS/MS measurements, while the inter-assay CV% was <9% and the Lower Limit of Quantitation was 0.5 nmol/L for both cortisol and cortisone. Cortisol and cortisone concentrations in saliva were determined with the same method as that for breast milk, but without the hexane-washing procedure.

Statistics

Conventional statistics

In total, 63 mother-infant pairs were included, of whom 55 pairs had valid GC levels for both mother and infant. Due to extremely high GC levels, one infant was excluded for cortisol analyses (n=54), and another infant was excluded for cortisone analyses (n=54).

GC levels were visualized by calculating mean (95% confidence interval (CI)) in 2-hour time windows. Additionally, linear mixed models (LMMs), which allow correcting for intra-individual measurements, were used to determine the slope of the increasing (i.e., 00:00-7:00) and decreasing (i.e., 7:01-23:59) part of the diurnal rhythm, in line with our previous study.(26)

Next, linear regression analyses were performed, for which seven additional mother-infant pairs were excluded because total sampling time was <8 hours and/or no samples were collected between 5:00-10:00 (i.e., sample collection around the morning peak), because this could interfere with the interpretation of the rhythm parameters. This resulted in 47 included mother-infant pairs.

Infant saliva and breastmilk cortisol and cortisone data were converted into rhythm parameters which, when taken into account together, will allow a full overview of HPA-axis rhythmicity:(36)

1. Area Under the Curve (AUC) with respect to the ground (AUCg) as well as increase (AUCi) were calculated by using the trapezoid rule as described by Pruessner et al. (2003).(37) AUC calculations were corrected for the total sampling time, since this differed between mothers. AUCg provides information on total GC exposure over the sampling time, while the AUCi is a measure of GC variability.
2. Maximum concentration measured, as a proxy for peak concentrations.
3. Time at which maximum concentration was measured, as a proxy for time of peak.

Associations between infant saliva rhythm parameters and increased HDS/HAS score, consultation at the POP outpatient clinic (as a proxy for vulnerability for maternal psychological distress), season at time of sampling (divided into two 4-month windows: 21/4 to 21/8 (summer) and 21/10 to 21/2 (winter); used as a parameter for light-dark exposure), and sex were analyzed. Season at time of

sampling analyses were repeated with time windows of 3 and 6 months, as well as by determining season at birth, divided into 3- , 4- and 6-month windows.

Additionally, the associations between breastmilk and infant saliva rhythm parameters were determined.

Lastly, the associations between maternal and infant raw GC levels, split up in 3 hour time intervals, were analyzed (n=54), by using LMMs.

Interactions with POP-clinic attendance were tested. No effect modification was found, and the data was therefore not stratified. Analyses assessing the possible influencing factors were repeated while only including mothers who did not attend the POP-clinic (n=40).

Sigmaplot analyses

Daily rhythmicity of cortisone and cortisol in the infants' saliva were assessed using Gaussian peak regression with Sigmaplot 14.0 software (SPSS Inc, Chicago, IL, USA). The data were best fitted (i.e., most optimal *P* value, least residuals and dependent on the least amount of variables) to the following regression formula, after testing single, double and triple peak formulas: y = a1*exp(-.5*((x-x1)/b1)^2) + a2*exp(-.5*((x-x2)/b2)^2), where a1 and a2 represent the estimates for the first and second peak heights, respectively; b1 and b2 represent the estimates for the full width at half maximum of the first and second peak, respectively (i.e. a measure of the broadness of the peak); x1 and x2 represents the estimates for the location (i.e. the timing along the 24h cycle) of the first peak and second peak, respectively. Intra-individual values were taken into account and grouped together through using the "shared parameters" function for the regressions.

GC rhythmicity was assessed separately for the following possible influencing factors: HADS-score (HDS and/or HAS < or ≥8), POP-clinic consultation (yes/no), season at sampling (21/4 to 21/8 and 21/10 to 21/2), sex (male/female), breastmilk AUCi (< or > p50), breastmilk AUCg (< or > p50). Subsequently, T-tests were used to calculated *P* values for differences in the timing of peaks (x1 and

x2). The estimates a and b were not compared since results were often found to be unreliable, in contrast to the x-estimate.

Results

Population description

Table 1 shows the population characteristics. Increased HDS and/or HAS scores were found in 10 mothers, half of whom were included at the Amsterdam UMC, location VUMC. Fifteen mothers were included at the POP-clinic, of whom 33.3% had clinically relevant depression (HDS) and/or anxiety (HAS) symptoms.

Infant GC rhythm

Regression analyses

Figures 1A and 1B show the infants' and breastmilk cortisol and cortisone levels over the day. A clear diurnal rhythm can be distinguished for both infant salivary and breastmilk GC levels. LMM analyses revealed that infant salivary as well as breastmilk GC levels significantly increased between 00:00-7:00 and significantly decreased between 7:01-23:59 [all *P* values <0.003].

Sigmaplot analyses

A significant biphasic cortisol and cortisone rhythm could be detected in the infants' saliva. The *P* values for overall fit as well as the placement of the peaks were *P*<0.0001 for both cortisol and cortisone. For cortisol, the first peak occurred at 6:53±1:01 (mean±SEM) and the second peak at 18:36±1:49. For cortisone, the first peak occurred at 8:50±1:11 and the second peak at 19:57±1:13. Analyses at an individual level could not be performed due to data constraints. It could therefore not be ruled out whether results represent a true biphasic rhythm in infants, or two separate groups of infants with a single morning or evening peak. Figure 1C and 1D show the cortisone rhythm for two individual infants, one with an adult-type rhythm (1C), and the other with a clear biphasic rhythm (1D).

Rhythm influencing factors

Regression analyses

Table 2 shows the associations between rhythm parameters and possible influencing factors. Male sex was associated with a lower salivary cortisol AUCi and a lower salivary maximum cortisol concentration. However, these associations were not present when salivary cortisone rhythm parameters were analyzed. No associations were found between rhythm parameters and other factors. Repeating season at time of sampling analyses with the other time windows did not reveal any associations either [data not shown].

Breastmilk maximum cortisol levels were positively associated with salivary cortisol AUCi and maximum levels in the infant (Table 3). Additionally, higher breastmilk cortisone AUCi, AUCg and maximum concentrations were associated with an earlier time of salivary maximum cortisone in the infant. No other associations were found.

Table 4 shows the associations between raw data of breastmilk and infant salivary GC concentrations, divided into 3-hour time intervals. A positive association was found for both cortisol and cortisone between 6:00-9:00, while no associations were found in the other time windows. Repeated analyses with Ln-transformed GC levels found similar results [data not shown].

When repeating the analyses while excluding the mother-infant pairs who attended the POP-clinic (n=7), small changes were found: an increased HDS/HAS score was associated with a higher maximum cortisol concentration, whereas sampling in the winter was associated with an earlier time of cortisol peak as well as a lower cortisone AUCi in the infants. The associations between male sex and a lower cortisol AUCi as well as between breastmilk cortisone AUCi and time of peak in the infants disappeared. Moreover, the association between breastmilk and infant GC concentrations collected between 6:00-9:00 disappeared as well.

Sigmaplot analyses

Table 5 shows the mean differences in time of peak for the studied possible influencing factors. Infants of mothers who attended the POP-clinic had a significantly earlier time of the second salivary cortisol peak. Time of the first salivary cortisol peak was earlier in infants with a breastmilk AUCi and AUCg >p50, and time of the second salivary cortisol peak was significantly earlier in infants with a breastmilk AUCg >p50. No differences were found in the timing of the salivary cortisone peaks.

When repeating the analyses while excluding mother-infant pairs who attended the POP-clinic (n=7), sampling in the winter was significantly associated with an earlier cortisol peak in the infants. None of the other associations changed.

Discussion

In this study, we have shown that full-term infants at a group level have a biphasic diurnal GC rhythm at the age of 1 month with peaks in the morning as well as in the evening. Increased risk for maternal psychopathologic complaints (increased HADS-score or POP-clinic consultation), season at sampling and sex were not associated with the infants' cortisol and cortisone rhythm parameters. Maternal GCs in breastmilk might be associated with the infants' GC rhythm, since a more variable breastmilk GC rhythm appears to be associated with an earlier time of maximum in infants. However, the sample size of the study was small and results were not consistent between cortisol and cortisone parameters, and should therefore be interpreted with caution.

The most striking finding of our study is the double peak that was found in the infant GC rhythm at a group level. A double peak has been described before,(8,38,39) although those peaks were not related to a specific time of day. Several explanations are possible for the presence of this double peak rhythm. First, the double peak was seen at a group level. Since analyses at the individual level could not be performed, it is possible that the biphasic rhythm was caused by two or more separate groups of infants, with some peaking in the morning, while others had a peak occurring in the evening. However, visualizing the data per mother-infant pair revealed that several infants appeared to have a double peak, with examples shown in Figure 1C and 1D. Alternatively, a double peak could be a part of the development towards an adult-type GC rhythm. Several studies have shown that an adrenal rhythm, with a peak in the afternoon/evening, might be present in utero.(10,11) After birth, under the influence of exogenous factors,(15) an adult-type adrenal rhythm develops. The fetal GC peak in the evening could therefore slowly disappear, and a morning peak might take its place. During this development, a transitional period might exist, in which the remnants of the fetal evening peak and the beginnings of an adult-type morning peak are both present. However, to test this hypothesis, longitudinal data are necessary.

Nevertheless, as far as we are aware, this is the first study to show the presence of a double peak at the age of 1 month. Ivars et al. (2015) have previously shown the presence of a significant GC rhythm at this age,(2) but did not report a double peak. Other studies have reported a later establishment of a GC rhythm in infants.(1,7,39,40) These differences in outcomes could be due to heterogeneity in statistical methods. Price et al. (1983) (6) defined a circadian rhythm as a higher value in the morning than in the evening, with a steady decline throughout the day. Santiago et al. (1996) (7) and Antonini et al. (2000) (40) considered a circadian rhythm to be present when afternoon and evening values were 83.5% or less of the morning concentration, whereas Ivars et al. (2015) (2) used a ratio of <0.8 between morning and evening levels to determine the presence of a rhythm. De Weerth et al. (2003) (1) used hierarchical linear modeling. All of these methods are based on the premise that a rhythm is present when there is a linear decline in GC concentrations. However, as we have shown, at a group level a second peak is present in the evening. Since this peak is on average lower than the morning peak, it is possible that a circadian rhythm is considered to be present according to these other methods, while the second peak is overlooked.

The possible influencing factors considered in this study were not significantly associated with the salivary infant GC rhythm, although when analyzing only mother-infant pairs who did not attend the POP-clinic, some effects of season of sampling were found on the timing of the cortisol, but not cortisone, peak of the infants. However, several associations were found between breastmilk and salivary infant GC parameters. The cortisol maximum in breastmilk was associated with more salivary cortisol variability, a higher maximum and earlier time of maximum in infants according to linear regression analyses, and higher breastmilk cortisol variability and total exposure were associated with earlier times of salivary cortisol peaks as analyzed by SigmaPlot. More cortisone variability, total exposure and a higher maximum in breastmilk were associated with an earlier salivary cortisone peak in the infants. Additionally, breastmilk and infant GC concentrations were correlated between 6:00-9:00 (i.e., during the morning peak). Whether these findings are due to a true association is unclear, since findings were not consistent between cortisol and cortisone parameters. Cortisol

concentrations especially are difficult to interpret in the saliva samples of infants, since 26% of the valid measurements were below the lower limit of detection (1 nmol/L). Cortisone levels were higher and did not reach the lower limit of detection, probably due to local conversion by 11β-HSD2.(30) Additionally, cortisone has been found to be more reliable than cortisol, at least in saliva and hair.(31,32) The results which use cortisone parameters are therefore likely to be more trustworthy. However, inert breastmilk cortisone would have to be converted to active cortisol in the infant. We have previously speculated that the gut microbiota might play a role in this.(41) Additionally, 11β-HSD1 is expressed in the human intestine, liver and in the SCN.(42-44) Even if only the analyses performed with cortisone parameters are reliable, it would seem that a more variable cortisone rhythm with a high peak in breastmilk could bring the time of the morning peak forward in infants, although these associations were only found in regression analyses. In light of our previous hypothesis, this could mean that GCs in breastmilk might aid in the transition from a fetal to an adult-type GC rhythm. The morning peak of GCs in breastmilk could have a role in this transition, since breastmilk and infant salivary GC levels were significantly correlated during this time-interval. The effects of breastmilk GCs could be caused by directly influencing GC concentrations in infant serum, although this is less likely due to the low absolute concentrations, or by acting as a signaling function in the infant's intestines (i.e., the "gut-brain axis hypothesis").(45,46) Alternatively, the associations might not be due to causality, but because another factor influences the maternal and infant HPA-axis in a similar fashion. Breastfeeding is associated with more responsive parenting (47) and increased maternal sensitivity (48) compared to formula feeding. Increased mother-infant synchrony caused by breastfeeding might therefore be a factor itself in influencing both maternal and infant GC rhythms.

This study has several strengths and limitations. First, our study's design enabled us to collect breastmilk and saliva samples at all hours of the day, with a total of 967 GC samples from 55 mother-infant pairs. Detailed analyses of infant and breastmilk rhythm could therefore be performed. Second, our analytical approach allowed for a detailed overview of infant GC rhythmicity, revealing a

double peak. Third, maternal distress was measured at time of sampling and its associations with the infants' HPA-axis activity could therefore be taken into account. Our study also has its limitations. Due to collection errors, quite some infant samples did not contain enough saliva for laboratory analyses. This meant that several mother-infant pairs had to be excluded because no valid samples were available around the time of the expected (maternal) peak (i.e., 5:00-10:00) or because total sampling time was not sufficient (i.e., <8 hours). However, these exclusion criteria attempted to reduce the chances of a bias, since GC levels of the excluded mother-infants pairs were likely to have lower maximum concentrations as well as AUC's. Additionally, our sample size of 55 mother-infant pairs is quite limited, and the number of subjects that attended the POP-clinic (n=15, 27.3%) and/or had an increased HADS-score (n=10, 18.2%) was also small, although the incidence of increased psychological distress in this study was comparable with the prevalence in the general population.(49) The statistical power therefore might have been too low to detect certain associations. It also required us to pool the available data, and individual as well as adjusted analyses were therefore not possible. On the other hand, with the exception of one study,(2) our sample size was bigger than other studies assessing HPA-axis development in early-life in term infants,(1,3,6-8,39,40). Moreover, although we studied several possible influencing factors, we did not take all determinants into consideration. For instance, no data was collected about daytime naps and sleeping times, while sleep has previously been associated with GC rhythmicity in infants.(1,8,50,51) Most of these associations were found in older infants than the ones included in this study, but an effect cannot be ruled out. However, daytime naps were associated with decreased cortisol levels immediately after the nap,(51) and they are therefore unlikely to explain the biphasic rhythm found in this study. Sleeping through the night was found to be associated with a more pronounced circadian rhythm,(1) but it has previously also been shown that the establishment of a diurnal GC rhythm precedes a sleep-wake rhythm.(3) Furthermore, the possibility of a selection bias cannot be excluded, because stressed mothers with infants who slept restlessly (indicative of a lack of rhythm) were probably less likely to participate, and it is therefore possible that the study population does

not reflect the general or the POP-clinic population. However, we did not collect data on mothers who were eligible for inclusion but opted out of participating, and a selection bias could consequently not be tested. Additionally, mothers who attended the POP-clinic might have had other reasons for not participating compared to mothers who did not attend the POP-clinic, which could have further skewed results. Lastly, a longitudinal study design would have enabled us a better understanding of HPA-axis development and which factors are of influence. However, we aimed to make this study as non-invasive as possible, and therefore decided to have mothers collect milk and saliva samples during one day only.

In conclusion, a biphasic GC rhythm appears to be present at a group level at the age of 1 month, with a peak in both the morning and the evening, which might be part of the developmental process towards an adult-type GC rhythm. Increased risk for maternal psychopathologic complaints (increased HADS-score or POP-clinic consultation), season at sampling and sex were not associated with infant GC rhythmicity in this study. However, breastmilk GC parameters might be associated with the infants' GC rhythm, which might be due to a causal signaling effect of breastmilk GCs, or because of an associative effect due to increased mother-infant synchrony. Although future studies should further elucidate HPA-axis development in early life, preferably with a longitudinal design and including a formula-fed control group, this exploratory study contributes to an increased understanding of this process, especially with regard to the role of breastmilk.

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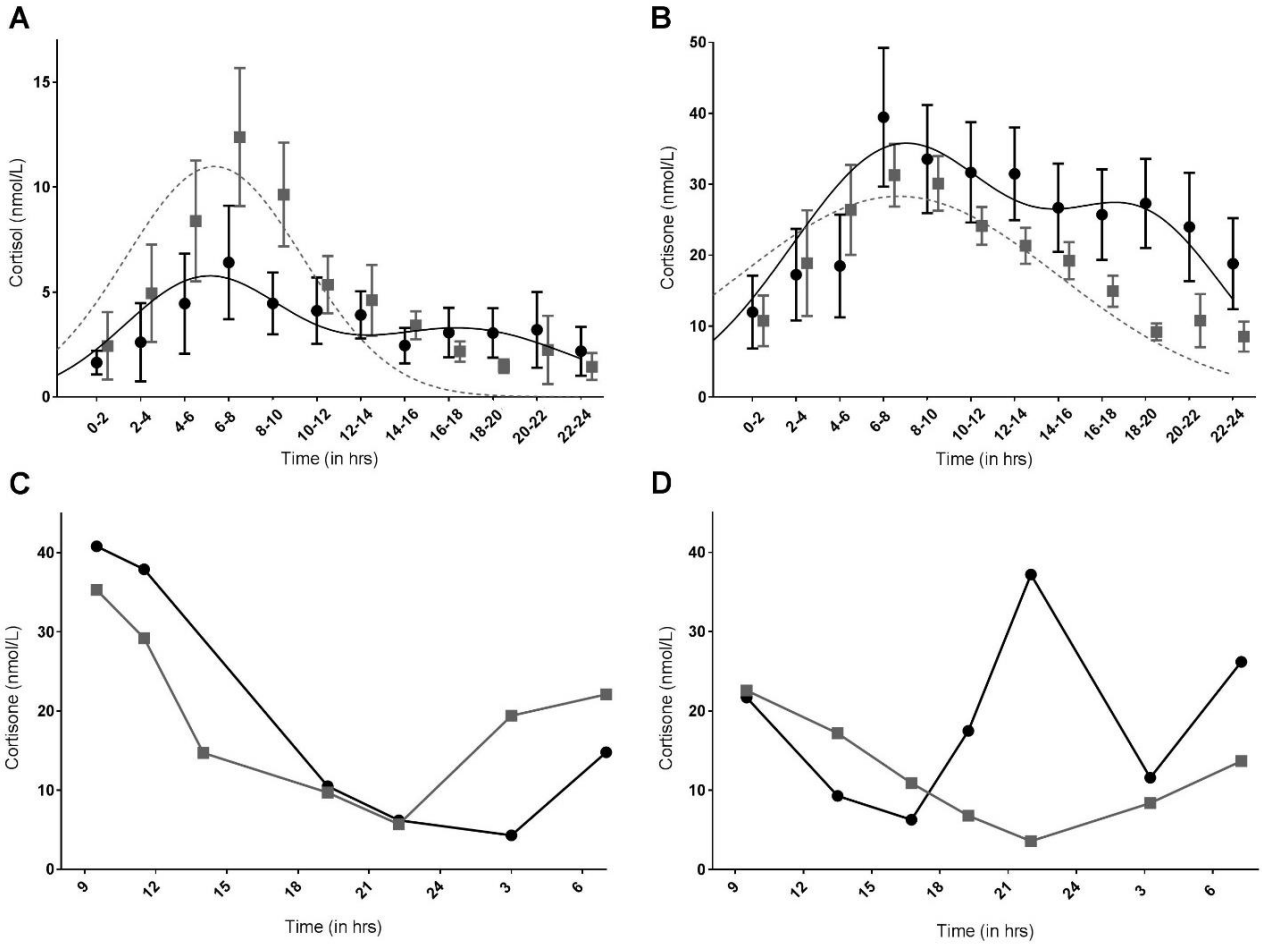

Figure 1: Cortisol (A) and cortisone (B, C & D) rhythms at a group level (A & B) and for two individuals (C & D), in infant's saliva (●) and breastmilk (■). The formula for cortisol (A) and cortisone (B) rhythms in infant's saliva as calculated by Sigmaplot is plotted as a continuous black line, the rhythm in breastmilk is plotted as a dotted grey line.

Table 1: Perinatal and maternal characteristics of the study population	
	n (%) or mean±SD
	n=55
Gestational age (weeks)	39.7±1.3
Birth weight - grams - SDS	 3550±467 0.2±0.9
Male sex	35 (63.6)
HAS/HDS >8 - Amsterdam UMC - OLVG hospitals, POP clinic	10 (18.2) 5 (12.5) 5 (33.3)
Consulted POP outpatient clinic	15 (27.3)
Season of birth: - between 4/21 and 8/21 - between 10/21 and 2/21	 17 (30.9) 14 (25.5)

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Table 2: Associations between GC rhythm parameters in the infants' saliva and possible influencing factors

			AUCi		AUCg		Maximum		Time of maximum	
		n	β (95%CI)	*P*	β (95%CI)	*P*	β (95%CI)	*P*	β (95%CI)	*P*
Cortisol	Increased HADS-score	47	0.3 (-1.1 to 1.6)	0.68	0.1 (-1.5 to 1.7)	0.90	1.9 (-3.3 to 7.1)	0.47	-1.1 (-5.3 to 3.0)	0.59
	POP-clinic consultation	46	-0.7 (-1.9 to 0.6)	0.28	-0.7 (-2.2 to 0.7)	0.31	-1.0 (-5.7 to 3.7)	0.67	-0.3 (-4.1 to 3.5)	0.88
	Season at sampling	29	-0.2 (-1.6 to 1.2)	0.75	-0.4 (-2.1 to 1.3)	0.62	-1.6 (-6.8 to 3.5)	0.52	-2.8 (-7.2 to 1.5)	0.19
	Male sex	47	-1.2 (-2.2 to -0.2)	0.02	-1.1 (-2.3 to 0.1)	0.07	-6.4 (-10.0 to -2.9)	0.001	-0.4 (-3.6 to 2.9)	0.83
Cortisone	Increased HADS-score	47	4.9 (-0.7 to 10.5)	0.08	3.7 (-3.8 to 11.2)	0.32	3.5 (-12.8 to 19.9)	0.67	-0.2 (-4.1 to 3.8)	0.94
	POP-clinic consultation	46	-1.4 (-6.7 to 3.9)	0.59	-2.0 (-9.0 to 5.0)	0.57	-12.3 (-26.8 to 2.2)	0.09	-2.4 (-6.0 to 1.2)	0.19
	Season at sampling	30	-4.5 (-10.0 to 1.0)	0.11	-5.5 (-12.8 to 1.9)	0.14	-8.8 (-26.1 to 8.4)	0.30	-0.3 (-4.3 to 3.7)	0.88
	Male sex	47	-0.9 (-5.4 to 3.6)	0.68	-0.9 (-6.9 to 5.0)	0.76	-5.5 (-18.1 to 7.1)	0.38	2.1 (-1.0 to 5.1)	0.18

Values represent β (95% CI) as analyzed with linear regression
Increased HADS-score: ≥8 on the HDS and/or HAS subscore
Season at sampling was divided into 4-month windows: 21/4 to 21/8 (summer) and 21/10 to 21/2 (winter)

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Table 3: Associations between GC rhythm parameters in the infants' saliva and breastmilk										
			Infants' saliva							
			AUCi		AUCg		Maximum		Time of maximum	
			β (95%CI)	*P*	β (95%CI)	*P*	β (95%CI)	*P*	β (95%CI)	*P*
Breastmilk	Cortisol	AUCi	0.2 (-0.04 to 0.4)	0.10	0.2 (-0.1 to 0.5)	0.19	0.7 (-0.2 to 1.6)	0.13	-0.4 (-1.1 to 0.3)	0.26
		AUCg	0.2 (-0.02 to 0.4)	0.08	0.2 (-0.1 to 0.4)	0.16	0.6 (-0.1 to 1.4)	0.11	-0.4 (-1.0 to 0.3)	0.27
		Maximum	0.1 (0.01 to 0.12)	0.02	0.1 (-0.01 to 0.1)	0.12	0.2 (0.02 to 0.4)	0.03	-0.2 (-0.3 to 0.02)	0.07
		Time of maximum	0.0 (-0.2 to 0.2)	0.90	0.1 (-0.1 to 0.4)	0.27	-0.2 (-1.0 to 0.5)	0.57	0.4 (-0.3 to 1.0)	0.25
	Cortisone	AUCi	0.4 (-0.2 to 1.0)	0.18	0.3 (-0.4 to 1.1)	0.41	1.5 (-0.1 to 3.1)	0.07	-0.4 (-0.8 to -0.1)	0.03
		AUCg	0.1 (-0.3 to 0.5)	0.66	0.1 (-0.5 to 0.7)	0.76	0.9 (-0.3 to 2.1)	0.13	-0.3 (-0.6 to -0.1)	0.02
		Maximum	0.2 (-0.1 to 0.4)	0.18	0.0 (-0.3 to 0.3)	0.91	0.4 (-0.2 to 1.1)	0.20	-0.2 (-0.4 to -0.04)	0.01
		Time of maximum	0.2 (-0.7 to 1.2)	0.65	0.8 (-0.4 to 2.1)	0.20	0.7 (-2.1 to 3.4)	0.63	0.4 (-0.3 to 1.0)	0.28
Values represent β (95% CI) as analyzed with linear regression AUCi: area under the curve increase, representing GC variability AUCg: area under the curve ground, representing total GC exposure										

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Table 4: Associations between breastmilk and infants' saliva GC concentrations per 3-hour time interval				
	Cortisol		Cortisone	
	β (95%CI)	*P*	β (95%CI)	*P*
0:00-3:00 (n=20/21)	-0.1 (-0.4 to 0.1)	0.35	-0.3 (-0.9 to 0.2)	0.21
3:00-6:00 (n=23)	0.2 (-0.1 to 0.5)	0.23	0.3 (-0.1 to 0.7)	0.14
6:00-9:00 (n=42)	0.2 (0.03 to 0.5)	0.03	0.7 (0.1 to 1.3)	0.03
9:00-12:00 (n=54/52)	0.0 (-0.3 to 0.3)	0.96	0.0 (-0.6 to 0.6)	0.94
12:00-15:00 (n=44)	0.1 (-0.04 to 0.3)	0.11	0.6 (-0.1 to 1.3)	0.09
15:00-18:00 (n=43)	-0.1 (-0.7 to 0.5)	0.78	-0.1 (-0.8 to 0.7)	0.88
18:00-21:00 (n=39)	0.1 (-0.7 to 1.0)	0.78	-0.2 (-1.4 to 1.0)	0.69
21:00-24:00 (n=35)	-0.3 (-1.4 to 0.7)	0.51	-0.3 (-1.6 to 1.0)	0.63
Values represent β (95% CI) as analyzed with linear mixed models, while adjusting for intra-individual measurements				

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Table 5: Mean differences (in hours) in time of peak for possible influencing factors				
	Cortisol		Cortisone	
	Peak 1	Peak 2	Peak 1	Peak 2
HADS-score	0:18±2:00	6:30±3:48	-0:54±6:06	0:36±6:36
POP-clinic consultation	-1:36±1:12	-5:12±1:42**	-1:42±2:12	-2:00±2:18
Season at sampling	-2:06±1:24	-2:30±2:48	-1:54±4:12	-2:12±3:42
Sex	-0:12±2:42	1:00±3:06	-1:54±3:12	-0:24±3:18
AUCi breastmilk	-3:12±1:30*	-3:12±3:18	-1:00±4:42	-0:18±5:18
AUCg breastmilk	-2:24±0:54*	-6:00±1:42***	-1:18±3:30	0:18±3:42

Values represent mean differences ± SEM in hours as tested with t-tests
* *P* value <0.05, ** *P* value <0.01, *** *P* value <0.001
HADS-score was dichotomized as <8 or ≥8 on the anxiety and/or depression subscore
Season at sampling was divided into 4-month windows: 21/4 to 21/8 (summer) and 21/10 to 21/2 (winter)
AUCi and AUCg of breastmilk were dichotomized as < and > p50

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